# The multifaceted functions of ATG16L1 in autophagy and related processes

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Noor Gammoh[1*]

[1] Cancer Research UK Edinburgh Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh, EH4 2XR, United Kingdom

*corresponding author: noor.gammoh@igmm.ed.ac.uk

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**Abstract**

Autophagy requires the formation of membrane vesicles, known as autophagosomes, that engulf cellular cargoes and subsequently recruit lysosomal hydrolases for the degradation of their contents. A number of proteins are required for autophagy that act to mediate the de novo biogenesis of autophagosomes and vesicular trafficking events. Of these proteins, ATG16L1 is a key player that has important functions at various stages of autophagy. Numerous recent studies have begun to unravel novel activities of ATG16L1, including **interactions with** protein**s** and lipid**s**, and how these mediate its role during autophagy and autophagy-related processes. Various domains have been identified within ATG16L1 that mediate its functions in recognising single and double membranes and activating subsequent autophagy-related enzymatic activities required for the recruitment of lysosomes. These recent findings, as well as the historical discovery of ATG16L1, pathological relevance, unresolved questions and contradictory observations, will be discussed here.

## Introduction

Autophagosomes are distinct double membrane vesicles that **form *de novo* to** encapsulate and deliver cellular components for lysosomal degradation in a process known as macroautophagy (hereafter referred to as autophagy). The activities of autophagy-related (ATG) proteins are essential for the recognition of degradation substrates and for autophagosome biogenesis (Gatica et al., 2018; Lamb et al., 2013). A subset of ATG proteins is also involved in so-called non-canonical autophagy or autophagy-related processes, which engage **pre-formed** single membrane vesicles (Galluzzi and Green, 2019). Regulation of ATG protein activities is essential to ensure the proper recruitment of lysosomes and to prevent aberrant degradation of cellular contents.

At the heart of autophagy, two ubiquitin-like conjugation machineries exist (Mizushima, 2019). These act to mediate the conjugation of ubiquitin-like proteins, **including** ATG12 **and** members of the ATG8 family of proteins, to ATG5 and the phospholipid phosphatidylethanolamine (PE), respectively. The two conjugation reactions require **the** common E1-like ubiquitin ligase ATG7, but distinct E2-like enzymes, ATG10 and ATG3, for the conjugation of ATG12 and ATG8 proteins, respectively. While no E3-like enzyme has been described for ATG12 conjugation, it is now established that the ATG5-ATG12 conjugate**, in complex with ATG16L1 (Mizushima et al., 2003),** harbours an E3-like activity during ATG8-PE conjugation (Fracchiolla et al., 2020; Lystad et al., 2019). Given that the ATG5-ATG12 conjugate is constitutively formed in cells, it can be predicted that the regulation of ATG8 conjugation, which is indicative of autophagic activity, occurs through the regulation of membrane availability, **lipid** modification, enzymatic activities and protein localisation **(Martens and Fracchiolla, 2020)**.

**ATG16L1 plays a crucial role during various steps leading to the conjugation of ATG8 proteins and accumulating recent data have revealed multifaceted activities of this autophagy essential factor (Fig. 1)**. This review will discuss in depth the function and regulation of ATG16L1 during autophagy and autophagy-related processes. The specific domains of ATG16L1 that mediate its functions as well as its pathophysiological relevance will also be examined.

## Brief outline of autophagosome formation

The characteristic double membrane nature of autophagosomes led to their initial identification as distinct organelles **(Hollenstein and Kraft, 2020; Kawabata and Yoshimori, 2020; Yang and Klionsky, 2010) (Fig. 2A)**. **Autophagy occurs at basal levels in cells and can be further induced upon various stimuli such as nutrient and oxygen deprivation as well as genotoxic and cytotoxic stresses (Antonucci et al., 2015; Kroemer et al., 2010; Mizushima et al., 2004; Russell et al., 2014). A number of protein complexes act to relay autophagy-stimulatory signals and engage in membrane modifications in order to facilitate the recruitment of the core ATG machinery to the pre-autophagosomal membrane (known as the phagophore or isolation membrane) (Box 1). Of these, the ULK complex, comprised of the ULK1 or ULK2 kinases and their adaptor proteins FIP200, ATG13 and ATG101, can be activated following autophagy-inducing stimuli (Wong et al., 2013) leading to the subsequent stimulation of VPS34 complex which catalyses the generation of phosphatidylinositol-3-phosphate (PI(3)P) on the phagophore (Russell et al., 2014). PI(3)P is required for the phagophore recruitment of the WIPI family of proteins (including WIPI1 and WIPI2) and the ATG5 complex (comprised of the ATG5-ATG12**

**conjugate bound to ATG16L1) (Itakura and Mizushima, 2010). The ATG5 complex can stimulate the lipid conjugation of ATG8 proteins, which include the LC3 (LC3A, LC3B and LC3C) and GABARAP (GABARAP, GABARAPL1 and GABARAPL2) sub-families (Johansen and Lamark, 2020; Mizushima, 2019).** The eventual fusion of lysosomes with autophagosomes (**forming autolysosomes**) requires components of the endocytic pathway and is key for the execution of a complete autophagic degradation (known as flux) **(du Toit et al., 2018; Loos et al., 2014)**.

**Brief outline of ATG8 conjugation on single membranes**

**Single membrane ATG8 conjugation (SMAC) (Durgan et al., 2020) has been reported and is a topic of emerging interest (Heckmann and Green, 2019; Sanjuan et al., 2007). SMAC involves the lipid conjugation of ATG8 proteins on already formed membranes and does not require membrane nucleation and growth, as is the case for autophagosome biogenesis (Fig. 2B). This non-canonical form of ATG8 lipidation can occur in the absence of the ULK and VPS34 complexes, but invariably requires the core ATG lipidation machinery, including ATG7 and the ATG5 complex (Florey et al., 2015; Florey et al., 2011; Martinez et al., 2011). Our current understanding of the underlying molecular mechanisms that mediate SMAC and the relevance of ATG16L1 will be discussed throughout this review.**

**Identification and structural organisation of ATG16L1/Atg16**

Identification

The identification of most ATG gene products (Atg1-Atg15) was performed in yeast using a screen for mutants that were unable to grow under nitrogen starvation conditions (Tsukada and Ohsumi, 1993). Atg16 (initially termed Apg16) was identified several years later using a yeast two-hybrid screen for yeast Atg12-interacting partners (Mizushima et al., 1999) and was followed by the identification of mammalian ATG16L1 (initially termed Apg16-like, Apg16L) by co-immunoprecipitation with ATG5 from mouse cells (Mizushima et al., 2003). Mammalian ATG16L1 and yeast Atg16 share high homology except for the presence of additional sequences within the C-terminal of ATG16L1 that are absent in its yeast counterpart (Fujioka et al., 2010) **(Fig. 3)**. Three main splice isoforms of **mouse** ATG16L1 were identified (α, β and □) that differ in sequences within their middle region as well as exhibit variable tissue expression patterns (Ishibashi et al., 2011; Mizushima et al., 2003).

Domains and structural organisation

ATG16L1 can be divided into three main domains that contribute to its distinct functions: an N-terminal region containing an ATG5 binding domain, a middle region containing a coiled-coil domain (CCD) and seven WD40 domains located within its C-terminal half (Fig. 1). The tertiary structures of these three domains have been individually determined providing a prediction of the overall structural organisation of the full length protein (Wilson et al., 2014). Mutations in individual domains of ATG16L1 enable the distinction of various forms of autophagy and autophagy-related processes (Fletcher et al., 2018; Gammoh et al., 2013).

**ATG16L1/Atg16 proteins likely exist in dimeric forms (Fujioka et al., 2010; Fujita et al., 2009).** Dimerisation of ATG16L1 occurs mainly through CCD sequences (Fujioka et al., 2010; Parkhouse et al., 2013) although dimerisation activities have also been detected in the N-terminal and WD40 domains of ATG16L1 (Bajagic et al., 2017; Kim et al., 2015). Size exclusion chromatography indicates that ATG16L1 exists in an approximately 800 kDa complex in cells, which is about three times the predicted size of an individual ATG16L1 dimer bound to two ATG5-ATG12 molecules (~250 kDa in size) (Ishibashi et al., 2011; Lystad et al., 2019; Mizushima et al., 2003). The two factors that are required for formation of this large molecular complex are binding to ATG5 and dimerisation via the CCD **(Mizushima et al., 2003; Saitoh et al., 2008)**. The precise components of this higher molecular weight complex are not fully understood. It is possible that solution-exposed amino acid residues within the CCD can mediate multimerisation of ATG16L1 as suggested by computational modelling (Kaufmann et al., 2014). This however remains to be experimentally confirmed as **the use of sucrose density gradients as alternative methods to predict complex sizes suggest that ATG16L1 is mainly found as a dimer in** cells (Fujita et al., 2009).

*N-terminus*

The N-terminus of ATG16L1 (residues 1-70) consists of an α-helix followed by a tail region and contains sequences required for binding to the ubiquitin-folds of ATG5 (Matsushita et al., 2007; Otomo et al., 2013). This interaction requires residues R35 and F46 of yeast Atg16 (corresponding to R24 and I36 of mammalian ATG16L1) and is essential for autophagy (Matsushita et al., 2007). The existence of additional residues in mammalian ATG16L1 (I17 and L21) required for ATG5 binding may suggest a divergence in the interaction between ATG16L1/Atg16 and ATG5/Atg5 between mammalian and yeast proteins (Kim et al., 2015; Mizushima et al., 2003). The N-terminus of ATG16L1 also harbours a membrane binding activity required for ATG8 lipidation (Lystad et al., 2019) (Fig. 1). The lipidation stimulatory activity of the ATG16L1 N-terminus **is** further supported by its **ectopic** plasma-membrane tethering, which was shown to be sufficient to drive LC3 lipidation in cells (Fujita et al., 2008). The E3-like ubiquitin ligase activity of the ATG5 complex is discussed further below.

*CCD and the middle region*

Coiled-coil regions are composed of helical structures that are known to contribute to protein-protein interactions. In ATG16L1, hydrophobic sequences within the CCD mediate its homodimerization (Fujioka et al., 2010), while solution-exposed residues can mediate binding to lipid and protein interactors, including phospholipids (Dudley et al., 2019) and Rab33B (Itoh et al., 2008). Interacting partners of yeast Atg16 CCD were also identified, including the PI(3)P effector Atg21 (Juris et al., 2015). Further downstream of the CCD within the middle region of ATG16L1, **sequences that mediate** interactions with WIPI2b (an isoform of WIPI2) **(Dooley et al., 2014),** FIP200 (Gammoh et al., 2013; Nishimura et al., 2013) **and lipids (Lystad et al., 2019) have been described**. These sequences are absent from yeast Atg16 and **their lengths differ between the ATG16L1 isoforms (highlighted in Fig. 3).**

The CCD is essential for autophagy and its deletion causes neonatal lethality in mice, which mimics knockout of core ATG genes (Saitoh et al., 2008). The importance of the middle region of ATG16L1 has been further highlighted by several observations. First, overexpression of ATG16L1 in some cell lines

leads to the inhibition of LC3 lipidation in a manner dependent on its middle region (amino acids 80-265 of mouse ATG16L1) (Fujita et al., 2008; Li et al., 2017a). Second, mutations in conserved and solvent-exposed residues of yeast Atg16 CCD, which do not contribute to the dimer-dimer interface, resulted in autophagy inhibition, suggesting a relevance of the CCD beyond dimer formation (Fujioka et al., 2010). Finally, the structurally-related and closely homologous ATG16L2 lacks LC3-lipidation-stimulating activity during canonical autophagy and can potentially exhibit inhibitory effects (Ishibashi et al., 2011; Khor et al., 2019; Wible et al., 2019). Elegant domain swapping experiments suggest that replacing the middle region of ATG16L2 with that of ATG16L1 can reconstitute LC3 lipidation to levels that are comparable to wild type ATG16L1 (Ishibashi et al., 2011). The absence of a localisation of ATG16L2 to phagophores is likely to explain its lack of autophagic activity as the ectopic localisation of its N-terminus to the plasma membrane can stimulate LC3 lipidation as seen with ATG16L1 (Fujita et al., 2008; Ishibashi et al., 2011). ATG16L2 can bind ATG5 and homodimerise, but it does not bind WIPI2b nor FIP200 and has reduced affinity to Rab33B, further highlighting the importance of the middle region of ATG16L1 (Dooley et al., 2014; Gammoh et al., 2013; Ishibashi et al., 2011). As residues that mediate these interactions are absent in yeast Atg16, it remains to be determined whether binding to these factors is sufficient to explain the lack of ATG16L2 autophagic activity.

*WD40 domains*

Seven WD40 domains span the C-terminal half of ATG16L1 and are absent in Atg16, indicating that they are dispensable for the role of ATG16L1 in canonical autophagy (Fletcher et al., 2018; Gammoh et al., 2013) (Fig. 1). Recent studies have begun to unfold a non-canonical **SMAC** function of this region (Fletcher et al., 2018). The WD40 domains of ATG16L1 form a seven-bladed propeller (Bajagic et al., 2017) and are involved in multiple protein-protein interactions, as discussed further below. Mice harbouring deletion in the WD40 domains of ATG16L1 exhibited no phenotypic abnormalities **or neonatal fatality** in contrast to the loss of ATG16L1 **CCD** (Rai et al., 2019; Saitoh et al., 2008). Bone marrow-derived dendritic cells from **mice lacking the WD40 domains of ATG16L1** showed defective MHC class II antigen presentation attributed to the non-canonical activity of ATG16L1 in facilitating LC3 lipidation on single membranes (Fletcher et al., 2018). **In addition, these animals exhibited an increased sensitivity to influenza A infection and reduced survival when compared to their control counterparts (Wang et al., 2020).** The exact mechanisms through which the WD40 domains mediate **SMAC** remain to be determined and likely involve yet unidentified interactions that include conserved residues within the top face of the WD40 domains (Fletcher et al., 2018).

**Function of ATG16L1 at different stages of autophagy**

Phagophore recruitment of the ATG5-ATG12 conjugate

Accumulating recent studies show that the phagophore recruitment of the ATG5 complex is mediated through ATG16L1 (Dudley et al., 2019; Juris et al., 2015; Li et al., 2017a) and is therefore a critical regulatory step during autophagosome biogenesis. The phagophore localisation of ATG16L1 requires components of the ULK complex, as well as VPS34-mediated PI(3)P production **(Fig. 2A)** (Itakura and Mizushima, 2010). Live-imaging studies suggest that ATG16L1 (bound to ATG5) and the ULK complex

concurrently localise to the growing phagophore (Koyama-Honda et al., 2013). This is likely mediated by the direct binding of ATG16L1 to the ULK complex component FIP200 (Gammoh et al., 2013; Nishimura et al., 2013). Genetic inhibition of FIP200 or deletion of the FIP200-binding domain of ATG16L1 abrogates puncta formation of the ATG5 complex **(Gammoh et al., 2013; Nishimura et al., 2013)**. The phagophore localisation of ATG16L1, but not the ULK complex, requires PI(3)P (Itakura and Mizushima, 2010), indicating that it may occur in a two-step manner, whereby initial recruitment of ATG16L1 is followed by its stabilisation on membranes.

The requirement of PI(3)P for the phagophore recruitment of ATG16L1 can be mediated by direct interaction of ATG16L1 to phosphoinositides, as well as binding to the PI(3)P effector WIPI2b (Dooley et al., 2014; Dudley et al., 2019). The requirement of multiple interaction partners may be important to prevent the aberrant recruitment of ATG16L1 on other membranes (Dudley et al., 2019; Ravikumar et al., 2010). Yeast Atg21 (a phosphoinositide-binding protein) was shown to bind the CCD of Atg16 (residues D101 and E102) (Juris et al., 2015). Genetic deletion of Atg21 in yeast does not fully disrupt starvation-induced Atg8 lipidation (Meiling-Wesse et al., 2004; Nair et al., 2010) but reduces autophagy and Atg16 phagophore recruitment under nutrient-rich conditions (Juris et al., 2015). Given that the $Atg16^{D101E102A}$ mutant is deficient in autophagy (Fujioka et al., 2010), it is possible that these sites mediate additional autophagic functions of yeast Atg16. **Interestingly, similar to the interaction between mammalian ATG5 and ULK complexes mediated by binding between ATG16L1 and FIP200 (Gammoh et al., 2013; Nishimura et al., 2013),** yeast Atg5 complex can also interact with the Atg1 (ULK1 homologue) complex but requires binding between Atg12 and Atg17 (a functional homologue of FIP200) (Harada et al., 2019). This suggests that diverged but multi-factorial mechanisms exist to target the ATG5 and Atg5 complexes in mammalian and yeast systems, respectively.

In mammals, PI(3)P, WIPI2b and FIP200 were shown to bind adjacent residues in the middle region of ATG16L1 **(Fig. 1)**. How these interacting partners crosstalk to mediate the efficient phagophore recruitment of ATG16L1 remains to be determined. Given that these three factors are dispensable for the role of ATG16L1 in mediating LC3 lipidation during mTORC1-independent autophagy or **on single membranes**, it is likely that additional means exist to mediate ATG16L1 localisation to membranes (Fletcher et al., 2018; Gammoh et al., 2013; **Lystad et al., 2019)**.

In addition, the displacement of ATG16L1 from membranes also appears to be important for LC3 lipidation with its regulation an open question. Enhancing the lipid-binding activity of ATG16L1 **within** its CCD, which is required for its phagophore recruitment, led to the inhibition of LC3 lipidation (Dudley et al., 2019). Similarly, unlike the plasma-membrane tethering of the N-terminal region of ATG16L1, tethering of a fragment that also includes the middle region (amino acids 1-249) to the plasma membrane inhibited LC3 lipidation (Park et al., 2016). These findings suggest that persistent membrane localisation of ATG16L1 can impose inhibitory effects on LC3 lipidation and its regulation is therefore crucial for autophagy.

Membrane nucleation

Beyond its role in recruiting the ATG5 complex to the phagophore, ATG16L1 can facilitate **phagophore growth** by localising to endocytic vesicles, which subsequently undergo fusion events **(Fig. 2A) (Puri et**

**al., 2013; Ravikumar et al., 2010)**. Recycling endosomes that are positive for Rab11 and ATG16L1 are thought to originate from the plasma membrane, thereby potentially contributing to a membrane source for autophagosome biogenesis (Puri et al., 2013; Ravikumar et al., 2010). However, the localisation of ATG16L1 to endosomes is not sufficient to drive LC3 lipidation, as transiently expressed ATG16L1 that predominantly localises to Rab11-positive recycling endosomes results in autophagy inhibition (Li et al., 2017a). Indeed, the recruitment of factors such as WIPI2b to Rab11a-positive endosomes are thought to facilitate the role of ATG16L1 during LC3 lipidation (Puri et al., 2018).

Similar to other membrane-fusion events, fusion of autophagy-related membranes appears to rely on the SNARE machinery. The homotypic fusion of ATG16L1-containing structures mainly requires VAMP7 and its accessory proteins, whereas heterotypic fusion events between recycling endosomes containing ATG16L1 or the transmembrane ATG protein ATG9, is mediated by VAMP3 (Moreau et al., 2011; Puri et al., 2013). Knockdown of either VAMP3 or VAMP7 reduces the levels of lipidated LC3 **(Moreau et al., 2011; Puri et al., 2013)**. An involvement of Annexin A2 has also been documented (Morozova et al., 2015). It is thought that Annexin A2 plays a part by influencing the phospholipid composition of ATG16L1-positive vesicles, as well as by supporting vesicle fusion events. Interestingly, Annexin A2 is also present at the plasma membrane and may thereby contribute to the plasma membrane recruitment of ATG16L1 **(Morozova et al., 2015)**.

Autophagosome maturation and ATG8 lipidation

The ATG5 complex components are all essential for its E3-like ubiquitin ligase activity that is required for the lipidation of ATG8 proteins *in vitro* and in cells. This involves the recruitment of the E2-like ligase ATG3 to the site of autophagosome biogenesis through direct binding **between ATG3 and** ATG12 (Otomo et al., 2013), as well as the ability of ATG16L1 to bind membranes through its N-terminal amphipathic helices (designated as helix 2) (Lystad et al., 2019). Helix 2 mutants of ATG16L1 form punctate structures similar to wild type protein, suggesting that its involvement in ATG8 lipidation occurs after its recruitment to phagophores. This N-terminal lipid-binding is required for **both single and double membrane lipidation of ATG8s** and may facilitate membrane remodelling events (Lystad et al., 2019). A distinct membrane-binding activity of ATG16L1 **located** downstream from its CCD **within the middle region termed β isoform lipid binding (Fig. 1)** has been also described and is required for PI(3)P-independent **SMAC** such as on perturbed endosomes (Lystad et al., 2019). **SMAC was shown to involve ATG8 conjugation to phosphatidylserine (PS) as well as to PE (Durgan et al., 2020) thereby raising the possibility that the β isoform lipid binding exhibits a capacity to mediate ATG8 to additional phospholipids (Lystad et al., 2019).** Interestingly, an isoform of ATG16L1 (ATG16L1α) lacks this second membrane-binding activity and, unlike its splice variant ATG16L1β, cannot support the lipidation of ATG8 during endosomal stress (Fig. 3) **(Lystad et al., 2019).** The tissue-specific expression relevance of these ATG16L1 isoforms remains largely unknown and elucidating these may help distinguish the roles of ATG16L1 during ATG8 lipidation on various membranes (Ishibashi et al., 2011).

Interactions with Rab GTPases and relevance in autophagosome-lysosome fusion

Rab GTPases are a family of guanine nucleotide (GTP)-dependent exchange factors involved in various stages of vesicle formation and trafficking **(Kiral et al., 2018; Zhen and Stenmark, 2015)**. They recruit specific downstream effectors when bound to GTP in their active state. Although their function is most extensively studied and linked to the endocytic pathway, accumulating data suggest they are also relevant during autophagosome biogenesis (Ao et al., 2014). Several Rab GTPases have been identified that can bind ATG16L1 and influence its subcellular localisation (Binotti et al., 2015; Itoh et al., 2008). The first indication that ATG16L1 can bind Rab GTPases came from a proteomic search for interacting partners of Rab33B, a Golgi-resident Rab GTPase with poorly defined function (Itoh et al., 2008). Subsequently, an interaction between ATG16L1 and Rab33A has also been described and shown to have autophagy-independent functions (discussed below) **(Ishibashi et al., 2012; Itoh et al., 2008)**.

The relevance of Rab33B-ATG16L1 interaction during autophagy remains poorly understood. RNAi-mediated knockdown of Rab33B does not significantly affect LC3 lipidation but the expression of constitutively active Rab33B appears to elevate the levels of lipidated LC3 and the autophagy receptor p62 (Itoh et al., 2008). In addition, expression of OATL1 (also known as TBC1D25), a guanosine triphosphatase–activating protein (GAP) for Rab33B, also enhanced LC3 lipidation and ATG16L1 puncta formation (Itoh et al., 2011). More detailed analyses showed that OATL1 and Rab33B can act by facilitating autophagosome-lysosome fusion (Itoh et al., 2011). Whether the interaction of Rab33B with ATG16L1 is relevant for its effects during lysosome fusion remains to be determined and may indicate that ATG16L1 can influence downstream events during autophagy. An additional involvement of the ATG5 complex in autophagosome-lysosome fusion was revealed upon the identification of tectonin beta-propeller repeat containing 1 (TECPR1) as an ATG5 binding partner (Chen et al., 2012). Mutating ATG5 binding sites in TECPR1 leads to an increase in GFP-LC3 puncta, consistent with a perturbed fusion between autophagosomes and lysosomes (Kim et al., 2015). ATG16L1 is excluded from the ATG5-TECPR1 complex and can be assumed to have an inhibitory effect on lysosome fusion in this setting (Chen et al., 2012).

**Regulation of ATG16L1 by post-translational modifications and stability**

Our current understanding highlights the importance of protein-protein and protein-lipid interactions in regulating the activities of most ATG proteins, including ATG16L1. Although many of these interactions appear constitutive and not affected by autophagy induction in cells (Dooley et al., 2014; Ganley et al., 2009; Nishimura et al., 2013), it remains plausible that post-translational modifications (PTMs) can play a role in regulating the activities of many ATG proteins. PTMs can provide a quick and reversible regulation of ATG proteins during autophagosome biogenesis. Indeed, a number of PTMs of ATG16L1 have been documented **(Fig. 1)**. For instance, phosphorylation of ATG16L1 (predominantly at S139) is stimulated during hypoxia/reoxygenation (H/R) treatment and requires the activity of casein kinase 2 (CK2) (Song et al., 2015). Mutating the putative CK2 phosphorylation site on ATG16L1 diminished its binding to ATG5 and inhibited autophagy **(Song et al., 2015). On the contrary**, phospho-mimetic mutants of this site or inhibiting its dephosphorylation **resulted in enhanced binding to ATG5 and restored autophagy (Song et al., 2015)**. This phosphorylation event can be regulated by an adjacent methylation of K151, which is decreased during H/R treatment, leading to elevated phosphorylation of S139 and enhanced binding to ATG5 (Song et al., 2018). **S278 of human ATG16L1 was also shown to be**

**phosphorylated by ULK1 (Alsaadi et al., 2019) although its phosphorylation by IκB kinase α (IKKα) has also been described (Diamanti et al., 2017)**. **Antibodies that specifically recognise the S278-phosphorylated ATG16L1 suggest that this site is modified under various conditions and can be used to detect its phagophore localisation (Tian et al., 2020).** Phosphorylation of S278 **by ULK1 is not essential for starvation-induced autophagy (Tian et al., 2020) but** is required for the recruitment of ATG16L1 to the site of invading bacteria during Salmonella infection (**Fig. 2C**) (Alsaadi et al., 2019). However, these stimulus-dependent modifications of ATG16L1 and the underlying molecular mechanisms that affect its autophagic activities remain to be further uncovered.

Regulation of protein stability of the core autophagy machinery could provide an alternative means to modulate their activities. ATG16L1, as well as other ATG proteins, have been identified as substrates for protease cleavage (Murthy et al., 2014; Norman et al., 2010). Increased susceptibility of ATG16L1 to caspase cleavage has been associated with its pathogenic variants in Crohn's disease (discussed below) **(Murthy et al., 2014)**. In addition, caspase cleavage and destabilisation of ATG16L1 is affected by IKKα- or ULK1-mediated phosphorylation of S278 (Alsaadi et al., 2019; Diamanti et al., 2017) and by its protein kinase A (PKA)-mediated phosphorylation (Zhao et al., 2019). Given that ATG16L1 overexpression can inhibit autophagy, it is possible that fine tuning of its levels may enable the regulation of its activity. Indeed, the E3 ubiquitin ligase Gigaxonin has been described to bind to the WD40 domains region of ATG16L1 and result in its degradation (Scrivo et al., 2019). In the absence of Gigaxonin, ATG16L1 levels are elevated, leading to its aggregation and autophagy inhibition, thereby imposing deleterious effects on neuronal health **(Scrivo et al., 2019)**. Interestingly, Gigaxonin overexpression can dramatically reduce ATG16L1 levels and restore autophagic activity in neurons **(Scrivo et al., 2019)**. This is consistent with the notion that low levels of some ATG proteins (such as ATG5) has been shown to be sufficient to drive autophagy in cells (Hosokawa et al., 2006), and raises the question whether the regulation of ATG protein stability is a means to modulate autophagy under all conditions.

**Relevance of WD40 domains interactions in non-canonical autophagy and cargo selection**

Unlike for autophagosome biogenesis, C-terminal sequences of ATG16L1 are vital for ATG8 lipidation on single membranes, such as perturbed endosomes or entotic bodies (Fletcher et al., 2018; Lystad et al., 2019). The underlying molecular mechanisms that mediate **SMAC** remain to be further explored, but it has been shown that they require sequences within the WD40 domains and the ATG16L1β-isoform specific C-terminal lipid binding region **(Fig. 1)** (Fletcher et al., 2018; Lystad et al., 2019). The physiological relevance of this non-canonical ATG8 lipidation also requires further investigation and might be uncovered by utilising transgenic mouse models that express WD40-deficient ATG16L1 (Rai et al., 2019; Wang et al., 2020) or lack specific ATG16L1 isoforms.

WD40 domains are known to mediate protein-protein interactions. Whilst multiple binding partners have been mapped to the WD40-containing C-terminal region of ATG16L1, they have not yet been confirmed to contribute to non-canonical ATG8 lipidation. **These WD40 domains binding partners include a subset of transmembrane-containing proteins, (Boada-Romero et al., 2013; Hu et al., 2016; Sun et al., 2017), ubiquitin (Fujita et al., 2013), component 3 (C3) (Sorbara et al., 2018) and ATP6V0C (Xu et al., 2019).** Many of these ATG16L1 interacting partners are dispensable for **starvation-induced**

**autophagy and are likely to have roles in autophagic cargo recognition or potentially** autophagy-independent functions.

The interacting partners of the WD40 domains of ATG16L1 suggest it might have a role in the clearance of bacteria by autophagy (known as xenophagy, **Fig. 2C**), such as the three different transmembrane-containing proteins TMEM59, TMEM74 and TMEM166 (also known as EVA1A) (Boada-Romero et al., 2013; Hu et al., 2016; Sun et al., 2017). Overexpression of any of these **proteins** was shown to induce LC3 lipidation. In the case of TMEM59, its overexpression together with ATG16L1 leads to the localisation of both proteins to *Staphylococcus aureus*-positive endocytic vesicles (Boada-Romero et al., 2013). Binding of the WD40 domains to ubiquitin has also been described and is required for the recruitment of ATG16L1 and LC3 to invading *Salmonella* in the absence of FIP200 (Fujita et al., 2013). **How FIP200 can compensate for requirement to bind ubiquitin, as well as any unknown factors,** remains to be investigated. Additional WD40-binding partners of ATG16L1 that may aid its recruitment to the site of bacterial infection during xenophagy include C3 and ATP6V0C (Sorbara et al., 2018; Xu et al., 2019). The interaction between ATP6V0C and ATG16L1 can be antagonised by the bacterial protein SopF as a mechanism to evade bacterial clearance by autophagy and thus enhance replication (Xu et al., 2019). Furthermore, the binding of C3 to ATG16L1 may have a role in mediating the survival of pancreatic cells in response to diabetogenic stress (King et al., 2019). Altogether, these findings highlight the role of ATG16L1 during xenophagy and suggest its relevance in cargo recognition and local activation of autophagosome biogenesis. Investigating the effects of smaller deletions or point mutations in the WD40 domains of ATG16L1, instead of deleting its entire C-terminus, may help to better understand the contribution of individual interacting partners.

### Autophagy-independent roles of ATG16L1

Multiple autophagy-independent roles of ATG16L1 have been reported and are defined here as those that do not **involve** any form of ATG8 lipidation (see Table 1). These functions of ATG16L1 may or may not require its interaction with the ATG5-ATG12 conjugate. Some of these autophagy-independent roles **in mammalian cells** are discussed below based on the requirement to bind to ATG5 **and additional activities in lower organisms are outlined in Box 2**.

#### ATG5-binding dependent ATG16L1 functions

Several studies have documented a role of ATG16L1 during exocytosis. ATG16L1, along with ATG5, has been shown to be involved in exosome production in a manner that includes the lipidation-independent recruitment of LC3 to vesicles (Guo et al., 2017). Exosomes can transport material from one cell to another and have been implicated in immune response, tumour cell metastasis and neurodegeneration **(Kalluri and LeBleu, 2020)**. **Genetic** inhibition of ATG16L1 or ATG5, but not ATG7, **in mouse embryonic fibroblasts or human breast cancer cells** disrupted the recruitment of LC3 to exosomes and their secretion **(Guo et al., 2017)**. Treating ATG5-null breast cancer cells with exosomes from wild type cells before their implantation in mammary pads in mice resulted in enhanced metastasis to the lungs compared to implantation of untreated wild type or ATG5-null cells (Guo et al., 2017). **The presence** of ATG16L1, along with the ATG5-ATG12 conjugate, in secretory vesicles **from cultured neuroendocrine**

**cells has also been reported and shown to depend on the binding of ATG16L1** to Rab33A (Ishibashi et al., 2012). This localisation of ATG16L1 is required for hormonal secretion in a manner that is independent of PI(3)P or components of the ULK complex (Ishibashi et al., 2012). ATG16L1 has also been found to be involved in lysosomal exocytosis, which is required to restrict cell-to-cell spreading of Listeria monocytogenes (Tan et al., 2018). Here, ATG16L1, along with the ATG5-ATG12 conjugate, acts to mediate plasma-membrane repair **dependently** on its WD40 domains, **but independently of other autophagy players** such as ATG3 **(Tan et al., 2018)**. An additional non-autophagic function of the ATG5 complex has been described in mediating interferon production during viral infection (Hwang et al., 2012). However, the underlying molecular mechanisms and whether this effect is mediated through regulation of the exocytic pathway remain unclear.

ATG5-binding independent roles of ATG16L1

Fewer non-ATG5-binding dependent roles of ATG16L1 have been described, which might be due to the fact that ATG16L1 is destabilised in ATG5-deficient cells (Dudley et al., 2019; Nishimura et al., 2013). One of these **ATG5-independent roles of ATG16L1 is the ability to suppress cytokine production mediated by the intracellular pattern recognition receptors,** Nod1 and Nod2 (Trindade and Chen, 2020), in response to bacterial infection (Sorbara et al., 2013). This effect of ATG16L1 is suppressed in the Crohn's disease variant ATG16L1T$^{T300A}$ (discussed below), potentially underscoring an autophagy-independent pathological role of this variant in inflammatory bowel disease (IBD) **(Sorbara et al., 2013)**.

**Link to pathophysiology**

The pathophysiological functions of ATG16L1 are likely mediated through both its autophagy-dependent and -independent activities. The first and most extensively studied disease-relevance of ATG16L1 was prompted by the discovery of its Crohn's disease-associated variant ATG16L1$^{T300A}$ (corresponding to β isoform residue number) **(Hampe et al., 2007)**. The link between ATG16L1 and intestinal inflammation was further confirmed in a number of studies, in which ATG16L1 was specifically deleted in either the intestine (Aden et al., 2018; Adolph et al., 2013; Matsuzawa-Ishimoto et al., 2017) or hematopoietic cells (Kabat et al., 2016; Saitoh et al., 2008; Samie et al., 2018), and together they demonstrated a protective role of ATG16L1 in IBD.

Despite its discovery as a susceptibility variant over a decade ago **(Hampe et al., 2007), research is still ongoing to determine the underlying molecular mechanism resulting in the association of ATG16L1$^{T300A}$ variant with increased susceptibility to Crohn's disease.** This threonine to alanine substitution falls within the WD40 domain of ATG16L1 that is in close proximity to its middle region and has been implicated in various functions of ATG16L1; however, the specific protein-protein or protein-lipid interactions that are affected by this variant are still undetermined (Boada-Romero et al., 2016). ATG16L1$^{T300A}$ may also be associated with additional pathological conditions, such as brain metastasis of lung cancer (Li et al., 2017b). Further genetic association studies may help to unravel additional diseases that are affected by this ATG16L1 variant.

The most **studied** molecular consequence of the ATG16L1$^{T300A}$ variant is its **enhanced** susceptibility to cleavage by Caspase-3 and thereby destabilisation (Murthy et al., 2014). Activation of Caspase-3 by

nutrient stress or death-receptor signalling stimulates ATG16L1$^{T300A}$ degradation and reduces autophagic response as well as clearance of bacterial infection **(Murthy et al., 2014)**. Mice that are hypomorphic for ATG16L1 expression or express ATG16L1$^{T300A}$ knock-in mutation exhibited altered intestinal microbiota and abnormalities in Paneth cells, specialised secretory cells that line up the intestinal epithelium and play an antimicrobial role in protecting the epithelium (Cadwell et al., 2008; Lassen et al., 2014; Lavoie et al., 2019).

Interestingly, variants of Nod2, an ATG16L1-binding partner, have also been associated with increased susceptibility to Crohn's disease. These Nod2 variants are thought to perturb its ability to recruit ATG16L1 to the site of bacterial infection, thereby affecting pathogen clearance (Travassos et al., 2010). **Both ATG16L1 and Nod2 can interact with immunity-related GTPase family M (IRGM) protein, another Crohn's disease-associated gene product (Chauhan et al., 2015). This complex formation is induced by microbial products and is required to supress pathogen-induced inflammation (Chauhan et al., 2015).** An additional binding partner of ATG16L1 that may be linked to its role in IBD is the anti-inflammatory protein A20, which binds ATG16L1 through its WD40 domains (Slowicka et al., 2019). Interesting, mice harbouring combined tissue-specific deletions of both A20 and ATG16L1, but not individual knockout, exhibit IBD-like phenotype, suggesting that both proteins can cooperatively regulate inflammation of the bowel (Slowicka et al., 2019). **Altogether, these Crohn's disease-associated interactors of ATG16L1 underscore its role in inflammatory response with the underlying mechanisms likely requiring its activities in autophagy and autophagy-related processes.**

## Conclusions and final remarks

As research continues to expand our knowledge of how ATG proteins function during autophagy, numerous questions remain unresolved. In terms of ATG16L1, recent studies have highlighted the importance of its various domains that engage in diverse steps of autophagosome biogenesis as well as during non-canonical **SMAC**. This raises the question of whether specific domain mutations of ATG16L1 could be used as tools to determine the physiological relevance of **canonical autophagy and non-canonical ATG8 lipidation.** Furthermore, it is also unclear what are the tissue-specific functions of the ATG16L1 isoforms. **In addition, h**ow ATG16L1 activity is regulated, i.e. whether there are any on/off switches, and what mediates its recognition of single membranes are other important aspects that warrant further investigation. **Finally,** the involvement of ATG16L1 in pathological conditions is also an area of ongoing research, and it remains to be addressed whether the underlying mechanisms involve its canonical or non-canonical autophagic functions, or instead require its largely unexplored non-autophagic activities.

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## Conflict of Interest

None declared.

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**Table 1. A summary of autophagy-dependent and -independent activities of ATG16L1.**

| Activity | Function | References |
|---|---|---|
| **Autophagosome formation** | | |
| Binding to FIP200 | Relaying upstream signalling from the ULK complex | (Dooley et al., 2014; Dudley et al., 2019; Gammoh et al., 2013; Nishimura et al., 2013) |
| Phagophore recruitment of ATG5-ATG12 (binding to WIPI2b, FIP200 and PI(3)P) | Specifying site of ATG8 lipidation | |
| E3-like ligase activity (ATG5-ATG12 and N-terminal lipid-binding) | ATG8 lipidation & autophagosome maturation | (Lystad et al., 2019; Otomo et al., 2013) |
| Localisation to endocytic vesicles | Phagophore growth | (Moreau et al., 2011; Puri et al., 2013; Ravikumar et al., 2010) |
| Multimer formation | Scaffolding phagophore growth | (Kaufmann et al., 2014) |
| Binding to Rab GTPases | Autophagosome-lysosome fusion? | (Itoh et al., 2008; Itoh et al., 2011) |
| **Non-canonical ATG8 lipidation (SMAC) & cargo recruitment** | | |
| Unknown protein/lipid binding partner within the WD40 domains | Recruitment to single membranes and subsequent ATG8 lipidation | (Fletcher et al., 2018) |
| C-terminal β isoform lipid-binding activity | ATG8 lipidation on single membranes | (Lystad et al., 2019) |
| WD40 domains interactions with: Ubiquitin, TMEMs, C3 and ATP6V0C | Relevance in recognising bacterial infections | (Boada-Romero et al., 2013; Fujita et al., 2013; Hu et al., 2016; Sorbara et al., 2018; Sun et al., 2017; Xu et al., 2019) |
| **Autophagy-independent** | | |
| Regulation of V1V0-ATPase activity via ATG5 binding | Exosome production | (Guo et al., 2017) |
| Rab33A binding | Hormone secretion | (Ishibashi et al., 2012) |
| Undefined WD40-dependent activity | Lysosome exocytosis and plasma membrane repair | (Tan et al., 2018) |
| Nod1 and/or Nod2 binding | Cytokine response | (Sorbara et al., 2013) |

## Boxes

### Box 1. A brief overview of autophagosome formation

Autophagy occurs at basal levels in cells and can be further induced upon various stimuli such as nutrient and oxygen deprivation as well as genotoxic and cytotoxic stress **(Antonucci et al., 2015; Kroemer et al., 2010; Mizushima et al., 2004)**. A number of signalling cascades can regulate autophagy (Russell et al., 2014). In the case of nutrient deprivation, the mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) pathways have been most widely studied with respect to their abilities to suppress or activate autophagy, respectively. These sensing complexes act to regulate autophagy by mainly phosphorylating members of the Unc-51-like kinase (ULK) complex, although additional regulatory mechanisms have been described (Wong et al., 2013). The ULK complex is important for the regulation of autophagy during nutrient sensing **(Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Mercer et al., 2009)**. Optimal kinase activity of ULK1 requires **autophosphorylation and** binding to its adaptor proteins and acts to activate downstream players including the VPS34 complex **(Wong et al., 2013; Zachari and Ganley, 2017)**. The VPS34 complex, comprised of the VPS34 lipid kinase, ATG14, Beclin-1 and VPS15 (Itakura et al., 2008), catalyses the production of PI(3)P on the phagophore, which is essential for the recruitment of subsequent ATG players (including WIPI2, ATG5 complex and ATG2 proteins) as well as for ATG8 lipidation (Dudley et al., 2020). The final **stages of autophagy involve autophagosome maturation and closure followed by lysosome fusion (forming the autolysosome) and** require the lipidation of the ATG8 family members **for its efficient execution (Johansen and Lamark, 2020; Nakamura and Yoshimori, 2017; Nguyen et al., 2016)**. Fusion between autophagosomes and lysosomes in the absence of ATG8 lipidation has been reported but occurs at a slower rate (Tsuboyama et al., 2016). The ATG8 family members are also involved in cargo and cargo receptor recognition although upstream ATG proteins **(such as FIP200)** may also play a role **(Kirkin and Rogov, 2019; Turco et al., 2019)**. **Lipidated ATG8s tethered to the outer autophagosomal membrane can be delipidated by the action of ATG4 proteases and recycled back to the cytoplasm (Maruyama and Noda, 2017).**

### Box 2. Relevance of ATG16L1 in autophagy-independent activities in non-mammalian organisms.

**Autophagy-independent roles of ATG16L1 have also been described in invertebrates. Drosophila mutants lacking Atg16 or expressing a truncation mutant that does not bind Atg5 exhibited reduced sensitivity to ethanol-induced sedation whereas flies lacking Atg7 or Atg3 showed no altered response to ethanol exposure (Varga et al., 2016). This increased resistance to ethanol is mediated by a defect in Corazonin production and secretion in Atg16 mutant flies (Varga et al., 2016). Individual or combined deletions of Atg16, Atg5 and Atg12 in the social amoeba *Dictyostelium discoideum* exhibited similar autophagy-related phenotypes as observed with other Atg deletions, including developmental abnormalities and reduced survival during nitrogen starvation (Karow et al., 2020). However, *D. discoideum* harbouring a combined triple deletion of Atg16, Atg5 and Atg12 showed increased defect in macropinocytosis of fluorescent-dextran and phagocytosis of yeast cells when**

**compared to Atg5 and Atg12 co-deletion alone, thereby suggesting additional non-autophagic and potentially redundant activities of these proteins (Karow et al., 2020). Altogether, these findings suggest that the autophagy-independent roles of Atg16 are also observed in varying species with the underlying molecular mechanisms poorly understood.**

**Figure Legends**

**Fig. 1. Domain organisation of ATG16L1.**

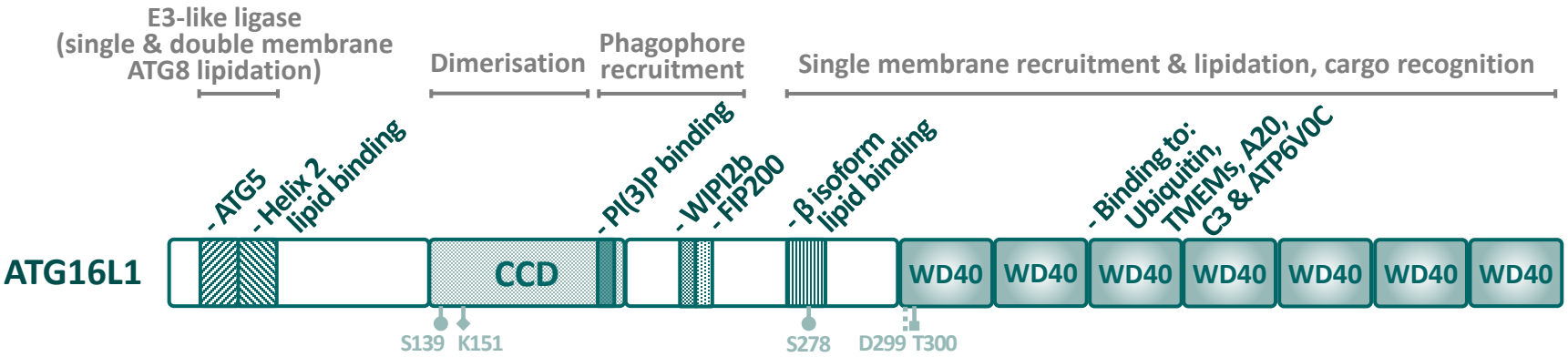
Major domains, interacting partners (protein and lipids) and activities of ATG16L1 are highlighted. Denoted above the protein are various regions mediating ATG16L1 activities **including those involved in double membrane autophagosome and single membrane vesicle ATG8 lipidations.** Interacting partners are also denoted diagonally above the protein. **PTMs are shown underneath the protein using oval arrowheads (S139 and S278 phosphorylation), diamond arrowhead (K151 methylation), square arrowhead (T300 Crohn's disease associated variant) and broken line (D299 caspase cleavage site).**

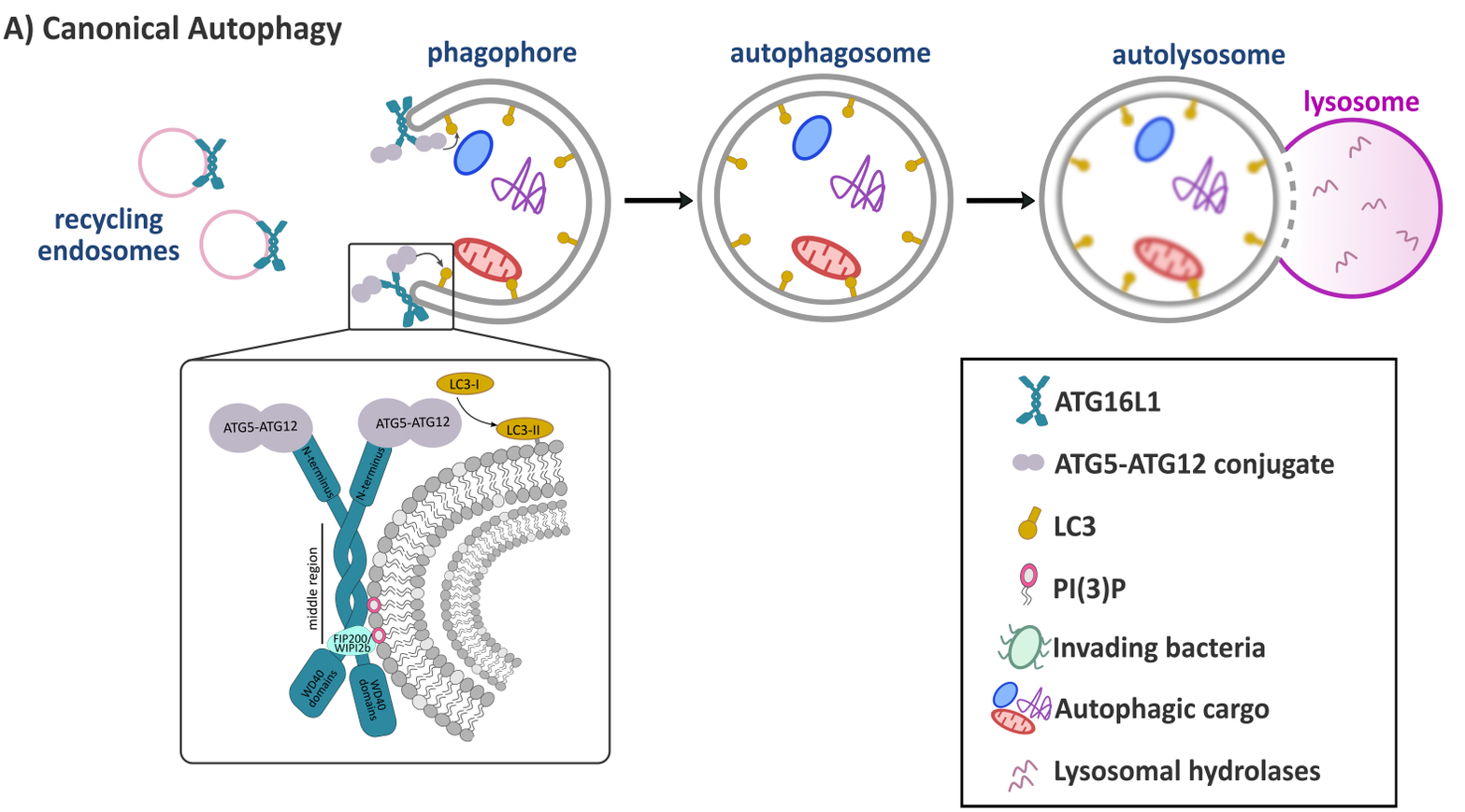
**Fig. 2. Relevance of ATG16L1 during autophagosome biogenesis and single membrane ATG8 lipidation.**

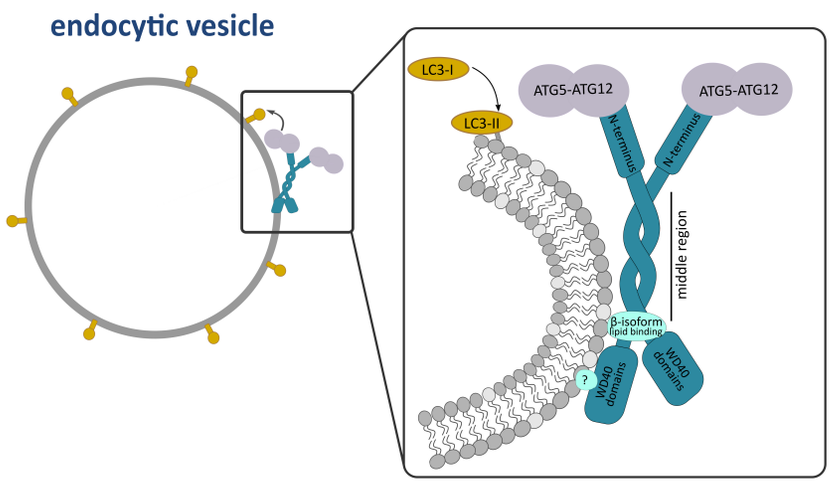
**A) Autophagosome biogenesis requires the formation of a precursor, double membrane structure termed the phagophore. ATG16L1 is recruited to the phagophore through its ability to bind FIP200, WIPI2b and PI(3)P. The localisation of ATG16L1 to endocytic vesicles (recycling endosomes) suggest its function in contributing to phagophore growth. These events facilitate ATG8 lipidation on the phagophore and contribute to autophagosome maturation, which subsequently fuse with the lysosome system forming an autolysosome. B) Single membrane ATG8 conjugation (SMAC) occurs on pre-formed vesicles such as entotic bodies, phagocytosed bacteria and perturbed endosomes. This requires various domains within ATG16L1 including N-terminal sequences (ATG5 and lipid binding), β isoform lipid binding and sequences within the WD40 domain. C) ATG16L1 is also involved in initiating autophagosome biogenesis at the site of invading bacteria during xenophagy (double membrane autophagosome) as well as initiating SMAC on phagocytosed bacteria (single membrane vesicles). Recognising invading bacteria by ATG16L1 requires various binding partners of the WD40 domains and is enhanced by S278 phosphorylation (pS278).**

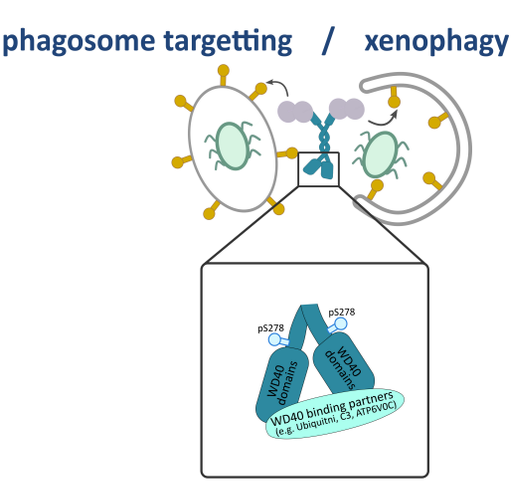
**Fig. 3. Comparison of ATG16L1-related proteins.**

Comparison of mammalian ATG16L1 (α, β and γ isoforms) and ATG16L2 as well as yeast Atg16 proteins. The relevant binding partners and dimerisation capacity are compared in the table. Not yet tested activities are marked with a question mark (?) and predicted activities based on sequence similarities are marked with a grey check mark (✓). Diagrams on the right depict major domains and relative length.


E3-like ligase
(single & double membrane
ATG8 lipidation)
Dimerisation
Phagophore
recruitment
Single membrane recruitment & lipidation, cargo recognition
- ATG5
- Helix 2
lipid binding
- PI(3)P binding
- WIPI2b
- FIP200
- β isoform
lipid binding
- Binding to:
Ubiquitin,
TMEMs, A20,
C3 & ATP6V0C
ATG16L1
CCD
WD40
WD40
WD40
WD40
WD40
WD40
WD40
S139
K151
S278
D299
T300


A) Canonical Autophagy
phagophore
autophagosome
autolysosome
lysosome
recycling endosomes
LC3-I
LC3-II
ATG5-ATG12
ATG5-ATG12
N-terminus
N-terminus
middle region
FIP200/ WIPI2b
WD40 domains
WD40 domains
ATG16L1
ATG5-ATG12 conjugate
LC3
PI(3)P
Invading bacteria
Autophagic cargo
Lysosomal hydrolases


B) Single Membrane ATG8 Conjugation (SMAC)
endocytic vesicle
LC3-I
LC3-II
ATG5-ATG12
ATG5-ATG12
N-terminus
N-terminus
middle region
β-isoform lipid binding
?
WD40 domains
WD40 domains


C) Recognition of Invading Bacteria
phagosome targetting / xenophagy
pS278
pS278
WD40 domains
WD40 domains
WD40 binding partners
(e.g. Ubiquitin, C3, ATP6V0C)

| | Dimer-isation | ATG5 | FIP200 | WIPI2 | CCD - lipid | Helix 2-lipid | β isoform lipid | Notes & distinctive features |
|---|---|---|---|---|---|---|---|---|
| **ATG16L1 α** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | X | Lacks C-terminal sequences required for single membrane ATG8 lipidation |
| **ATG16L1 β** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Supports single & double membrane ATG8 lipidation |
| **ATG16L1 γ** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Longest ATG16L1 isoform mainly expressed in the brain & heart |
| **ATG16L2** | ✓ | ✓ | X | X | ? | ? | ? | Divergence of unspecified middle region sequences results in autophagic inactivity |
| **Atg16 (Yeast)** | ✓ | ✓ | N/A | N/A | ? | ? | N/A | Lacks C-terminal sequences including the WD40 domains |